

## A new black fly species from Brazil, closely related to *Simulium guianense* Wise (Diptera, Simuliidae), revealed by morphology and DNA barcoding

NEUSA HAMADA<sup>1</sup>, MATEUS PEPINELLI<sup>2</sup>, ALINE MATTOS-GLÓRIA<sup>1,3</sup>

& SERGIO LUIZ BESSA LUZ<sup>4</sup>

<sup>1</sup>Coordenação de Pesquisas em Entomologia, Caixa Postal 478, 69011-970, Manaus, AM, Brazil. E-mail: nhamada@inpa.gov.br

<sup>2</sup>Laboratório de Entomologia Aquática, Departamento de Hidrobiologia, Universidade Federal de São Carlos, Caixa Postal 676, 13565-905, São Carlos, SP, Brazil. E-mail: mateuspepi@yahoo.com.br

<sup>3</sup>Divisão de Curso em Genética, Conservação e Biologia Evolutiva, Instituto Nacional de Pesquisas da Amazônia, Caixa Postal 478, 69011-970, Manaus, AM, Brazil. E-mail: mattosaline@hotmail.com

<sup>4</sup>Centro de Pesquisa Leônidas e Maria Deane - Biodiversidade em Saúde, Fundação Oswaldo Cruz, Rua Teresina n. 476, Adrianópolis, 69.057-070, Manaus, AM, Brazil. E-mail: sergioluz@amazonia.fiocruz.br

### Abstract

The male, female, pupa and larva of *Simulium litobranchium* n. sp. are described and illustrated. This new species has 12 gill filaments, as do *S. duodenicum* Pepinelli, Hamada & Trivinho-Strixino, *S. guianense* Wise, *S. hirtipupa* Lutz, *S. perplexum* Shelley, Maia-Herzog, Lunas Dias & Couch and *S. scutistriatum* Lutz, but it can be distinguished from these other species by a combination of characters observed at the pupal and adult stages. DNA barcoding showed that *Simulium litobranchium* n. sp. exhibits more than 4% nucleotide divergence in cytochrome oxidase I from three other closely related species in the Neotropical subgenus *S. (Thrysopelma)*. The new species was collected in the Brazilian states of Goiás and Minas Gerais and inhabits rivers 30–40 m in width in the Paraná River hydrographic basin. Females were not observed engaging in anthropophilic behavior.

**Key words:** aquatic insects, taxonomy, COI, genetic distance, sibling species

### Introduction

Shelley *et al.* (2002) described morphological variation in larvae of *S. guianense* Wise collected in the state of Goiás, denominating them as atypical. They reported that the only reliable character to distinguish them from the typical *S. guianense* is the presence, in the larva, of a pair of raised tubercles on the dorsolateral surface of abdominal segments 1 to 6. They also reported differences in wing vein setation of both genders in comparison with the typical form of this species. At the pupal stage they did not observe any difference.

*Simulium guianense* is the main vector of *Onchocerca volvulus* (Leuckart) in locations in the Brazilian and Venezuelan Amazonian where onchocerciasis is confined, and this simuliid is widely distributed in Brazil. It is therefore very important to establish the morphological boundaries of this species.

Molecular methods have been used to understand relationships between populations or between closely related species. Among the available molecular tools, sequencing of gene fragments allows direct evaluation of DNA polymorphism, providing additional data to make inferences about relationships between specimens, populations, and species. Different segments of mitochondrial and nuclear DNA evolve at different rates. Regions with rapid evolution are adequate for studying closely related taxa, while more conservative regions are appropriate for comparing more divergent taxa (Avise 1994). Molecular methods of analysis have permitted many innovations in insect systematics (Roderick 1996; Caterino *et al.* 2000).

One molecular approach that has been effective in identifications and in revealing cryptic species of black flies is DNA barcoding (Rivera & Currie 2009). Since DNA barcoding was proposed by Hebert *et al* (2003) as a reliable, quick and cost-effective identification system for the entire animal kingdom, it has been widely applied to help resolve myriad taxonomic problems, including speeding up the discovery of new species thus aiding the fight against biodiversity loss (see May 2009 special issue of *Molecular Ecology Resources* on “Barcode of Life”).

The objective of the present study is to demonstrate that the atypical form of *Simulium guianense* *sensu* Shelley *et al.* (2002) actually represents a distinct undescribed species closely related to *S. guianense*. The two species are shown to differ both morphologically and molecularly (mtDNA - COI sequences).

## Material and methods

Specimens of the new species were collected in Montividiu and Delfinópolis Municipalities, in Goiás and Minas Gerais states, respectively. In Montividiu, two rivers were sampled: the Montividiu ( $17^{\circ}26' S$ ,  $51^{\circ}10' W$ , 779 masl) and Ponte de Pedra Rivers ( $17^{\circ}10' S$ ,  $50^{\circ}50' W$ , 671 masl). In Delfinópolis the specimens were collected in the Santo Antônio River ( $20^{\circ}15' S$ ,  $46^{\circ}51' W$ , 726 masl).

The techniques for collection, rearing, dissection, and terminology are those detailed by Adler *et al.* (2004) and Pepinelli *et al.* (2005). Description of the thoracic pattern was based on specimens recovered from alcohol, using the technique of Sabrosky (1966); after drying, insects were glued to a triangle and pinned. Images illustrating this paper were obtained directly from specimens using a Nikon digital camera attached to a dissecting microscope and an image-capture system (Olympus Q-color 5), attached to a compound microscope. Many plates were made by stacking photographs to obtain three-dimensional images, using open software (Combine ZM 2007).

For molecular analyses, black fly specimens (larvae, pupae and adults) were sampled in five streams and rivers in three Brazilian states (Table 1). In this study we analyzed 48 specimens in four species, all of which had COI sequences amplified (> 650 bp) and analysed using the DNA barcoding approach. We used only a small piece of tissue from each specimen to extract the DNA. All the remnants were kept as voucher specimens and are deposited in the collection of Laboratório de Entomologia Aquática at the Universidade Federal de São Carlos, São Paulo, Brazil. The primers LepF (5-ATTCAACCAATCATAAAGATATTGG-3) and LepR (5-TAAACTCTGGATGT CCAAAAAATCA-3) amplified the target 658-bp fragment of COI. Sequences were obtained by using either ABI 377 or ABI 3730 sequencers (Applied Biosystems) and Big Dye v3.1 terminators mix (the sequencing protocol is available at: [http://www.dnabarcoding.ca/CCDB\\_DOCS/CCDB\\_Sequencing.pdf](http://www.dnabarcoding.ca/CCDB_DOCS/CCDB_Sequencing.pdf)).

**TABLE 1.** List of black fly species, location of collection sites and number of specimens and populations (pop) analyzed. Maximum and mean intraspecific values of genetic divergence (Kimura 2-parameter pairwise distances) are shown.

Species	Locality (State and city)	No. of specimens	No. of populations	%divergence [max. (mean)]
<i>Simulium duodenicum</i>	SP, Joanópolis	13	1	0.31 (0.09)
<i>Simulium guianense</i>	SP, Terra Roxa and Guaira/ Miguelópolis	13	2	2.67 (1.57)
<i>Simulium litobranchium</i>	GO, Montividiu	3	1	0
<i>Simulium scutistriatum</i>	MG, Santa Bárbara, Caraça (Cascatinha and Taboões)	18	2	1.08 (0.23)

Opposing electropherograms were assembled into contigs, reconciled, and aligned using SEQUENCER (Gene Codes, Ann Arbor, MI). Sequence information was entered into the Barcode of Life Database (BOLD, [www.barcodinglife.org](http://www.barcodinglife.org)) along with an image and information on data collection for each voucher specimen.

The detailed specimen records and sequence information, including trace files, are available on the BOLD in two project files (Barcode of Neotropical Black Flies (Simuliidae) and Barcode of Neotropical Black Flies 2). Kimura's two-parameter model of base substitution (Kimura 1980) was used to calculate genetic distances in MEGA 4.0 software (Tamura *et al.* 2007), and NJ trees were produced by using BOLD and MEGA 4.0 software.

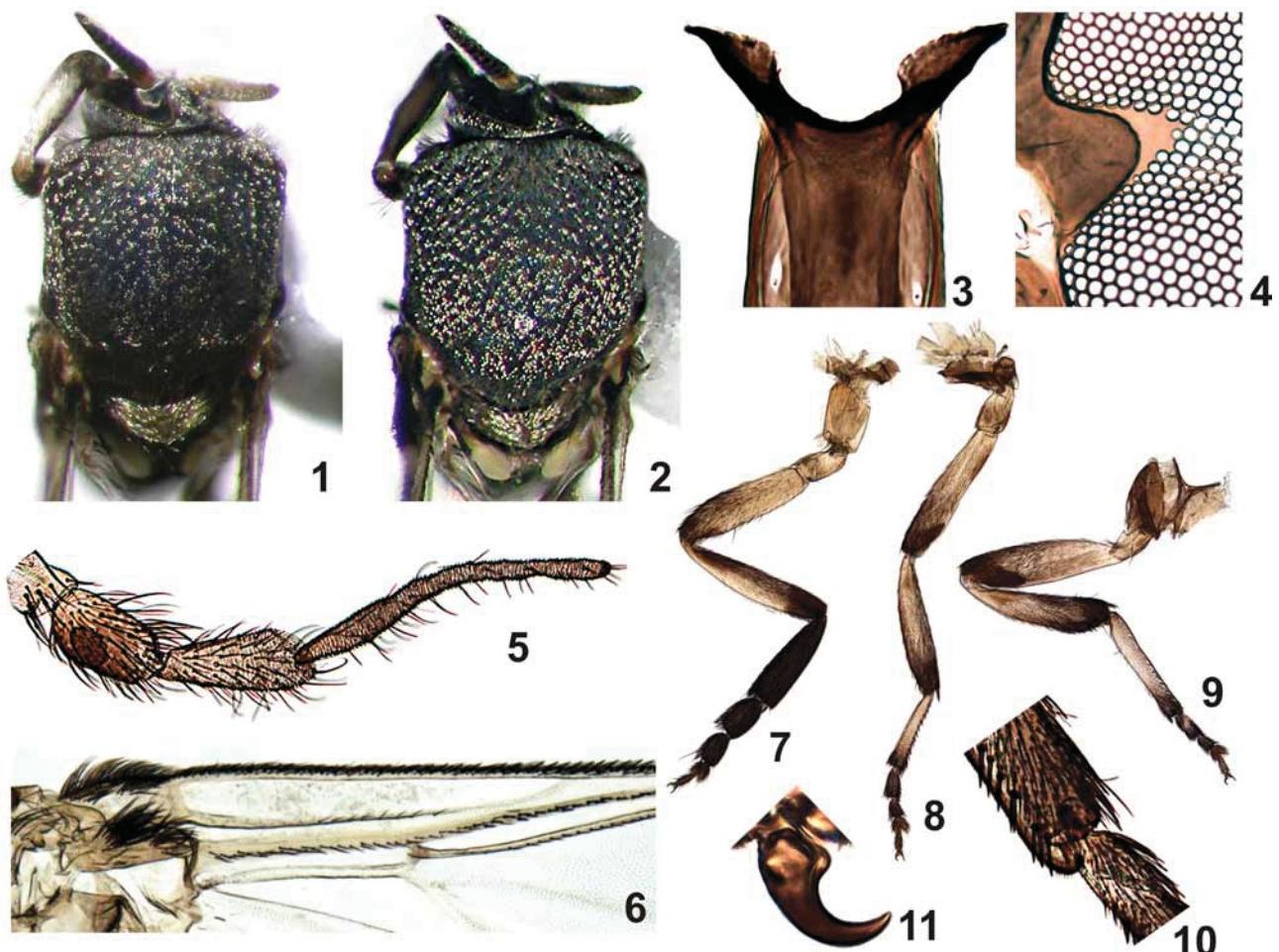
The holotype and paratypes are deposited in the Invertebrate Collection of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, Amazonas, Brazil; other paratypes are deposited in Museu de Zoologia de São Paulo. Voucher specimens of other Simuliidae species are deposited at INPA.

## Description

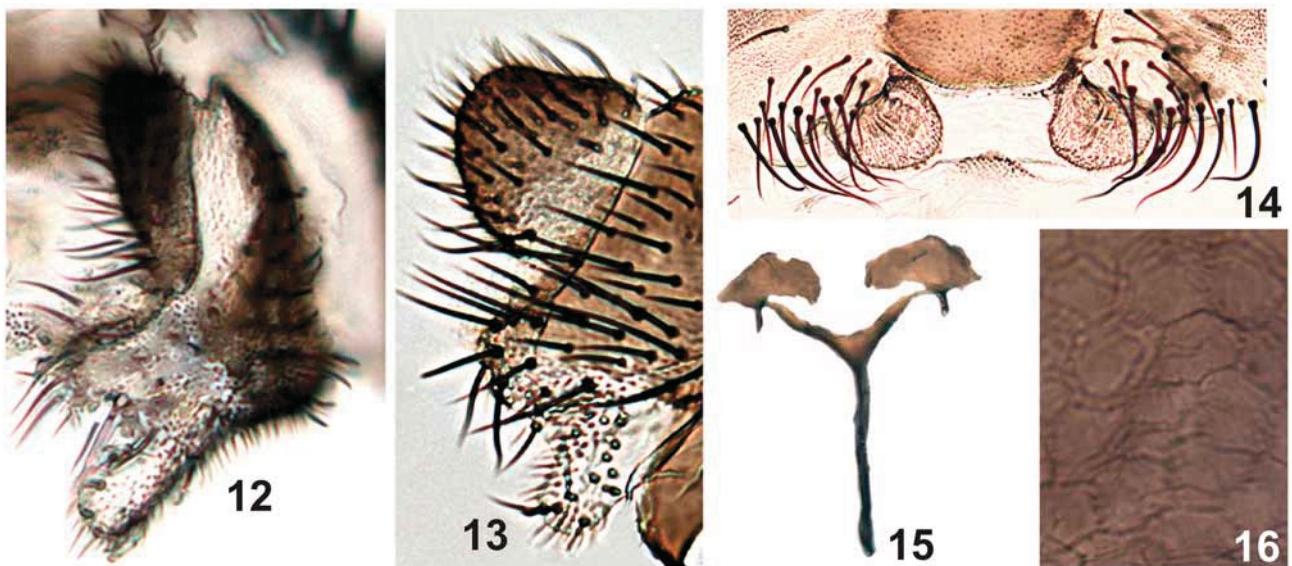
### *Simulium litobranchium* Hamada, Pepinelli, Mattos-Gloria & Luz New Species

Figs 1–16, 20–32, 35, 38, 41, 44–52, 57–70

**Diagnosis.** Female: with internal side of the anal lobe concave in the medial region, forming two short projections of similar width; presence of hair-like setae on the Sc. Male: ventral plate subrectangular, with median anterior projection; apical region of the projection larger than its base; anterolateral region of the ventral plate elongated. Pupa: with 12 short, thick, rigid filaments, without spicules in annular arrangement, with tips pointed and sclerotized; thorax without tubercles on the dorsal anterior region; dorsal posterior region with tubercles mostly pointed. Larva with paired dorsal abdominal tubercles, varying from weak to strongly



**FIGURES 1–11.** Female of *Simulium litobranchium* n. sp. (Diptera: Simuliidae). 1. Scutum, dorsal view, anterior illumination. 2. Scutum, dorsal view, posterior illumination. 3. Cibarium. 4. Fronto-ocular triangle. 5. Maxillary palp. 6. Wing base. 7. Fore leg. 8. Middle leg. 9. Hind leg. 10. Calcipala and pedisulcus. 11. Tarsal claw from middle leg.



**FIGURES 12–16.** Female of *Simulium litobranchium* n. sp. (Diptera: Simuliidae). 12. Cercus and anal lobe *in situ*, ventral view. 13. Cercus and anal lobe, mounted between slide and coverslip, lateral view. 14. Hypogynial valves, ventral view. 15. Genital fork. 16. Cuticular microspines of spermatheca.

developed on the 1<sup>st</sup> to the 5<sup>th</sup> or 6<sup>th</sup> segment; gill after histoblast dissection, without spicules in annular arrangement.

FEMALE (Figs 1–16). General color black; body mean length (from anterior region of head to abdomen tip) 1.8 mm (SD = 0.08, n = 5); lateral thorax mean length (from neck to wing base) 0.6 mm (SD = 0.04, n = 5). Wing mean length 2.1 mm (SD = 0.1, n = 11); mean width 1.0 mm (SD = 0.03, n = 11).

Frons brown, covered with short, golden hairs; clypeus brown with silver pruinosity, covered with short, golden hairs; frons longer than wide; fronto-ocular suture absent, fronto-ocular triangle as in Figure 4. Antenna mean length 0.53 mm (SD = 0.03 n = 7); scape, pedicel and first flagellomere brownish yellow, subsequent flagellomeres increasingly dark brown. Palpus dark brown; sensory vesicle length less than half the length of palpomere III (Fig. 5), palpomere V 2.6 times as long as palpomere III and 2.2 times as long as palpomere IV (Fig. 5). Mandible with 5–10 (mean = 8.1, SD = 2.0, n = 9) weak external serrations and 18–31 (mean = 29.1, SD = 4.5, n = 9) internal teeth. Lacinia with 24–29 (mean = 26.4, SD = 1.5, n = 9) retrorse teeth. Cibarium with sclerotized cornuae (Fig. 3); area between the cornuae sclerotized with small serrations. Scutum black, covered with golden hairs, distributed in small groups (Figs 1, 2). Anepisternum and katepisternum brown with silver pruinosity. Scutellum brown, with gold and brown hairs; postnotum brown, with silver pruinosity. Wing Costa with spiniform and hair-like setae, Sc and R with hair-like setae (Fig. 6). Foreleg (Fig. 7) with coxa, trochanter and femur yellowish brown; tibia with external margin whitish, except 1/5 distal area that is dark, internal margin brown; basitarsus and tarsomeres I–IV dark brown. Middle leg (Fig. 8) with coxa and trochanter yellowish brown; femur and tibia yellowish brown with distal region dark brown; basitarsus mostly yellowish brown, except a small portion in the distal end dark brown; tarsomere I–IV dark brown. Hind leg (Fig. 9) coxa, trochanter and femur brown, tibia with basal 1/3 whitish and 2/3 dark brown; and basal 3/4 of basitarsus whitish, distal 1/4 dark brown; tarsomeres I–IV dark brown; calcipala longer than wide, reaching pedisulcus (Fig. 10). Tarsal claws curved, without subbasal tooth (Fig. 11). Femora and tibiae of the middle and hind leg with narrow scale-like setae and thin setae. Basal fringe, in the abdomen with long golden-whitish and brown hairs. Tergite II with lateral silver pruinosity; tergites VI–VIII with varnish-like dorsal plate. Cercus subtriangular, anal lobe with internal area concave medially, forming two short projections, anterior projection longer than the posterior one (Figs 12, 13). Hypogynial valves (Fig. 14) not sclerotized, rounded, with microtrichias. Genital fork (Fig. 15) with stem long, lateral arms well developed forming subtriangular space; lateral plate well developed (Fig. 15) and apodemes strong. Spermatheca subspherical, with cuticular microspines distributed in nearly pentagonal pattern (Fig. 16); spermathecal duct and area of attachment unpigmented.



17



18



19



20



21



22



23

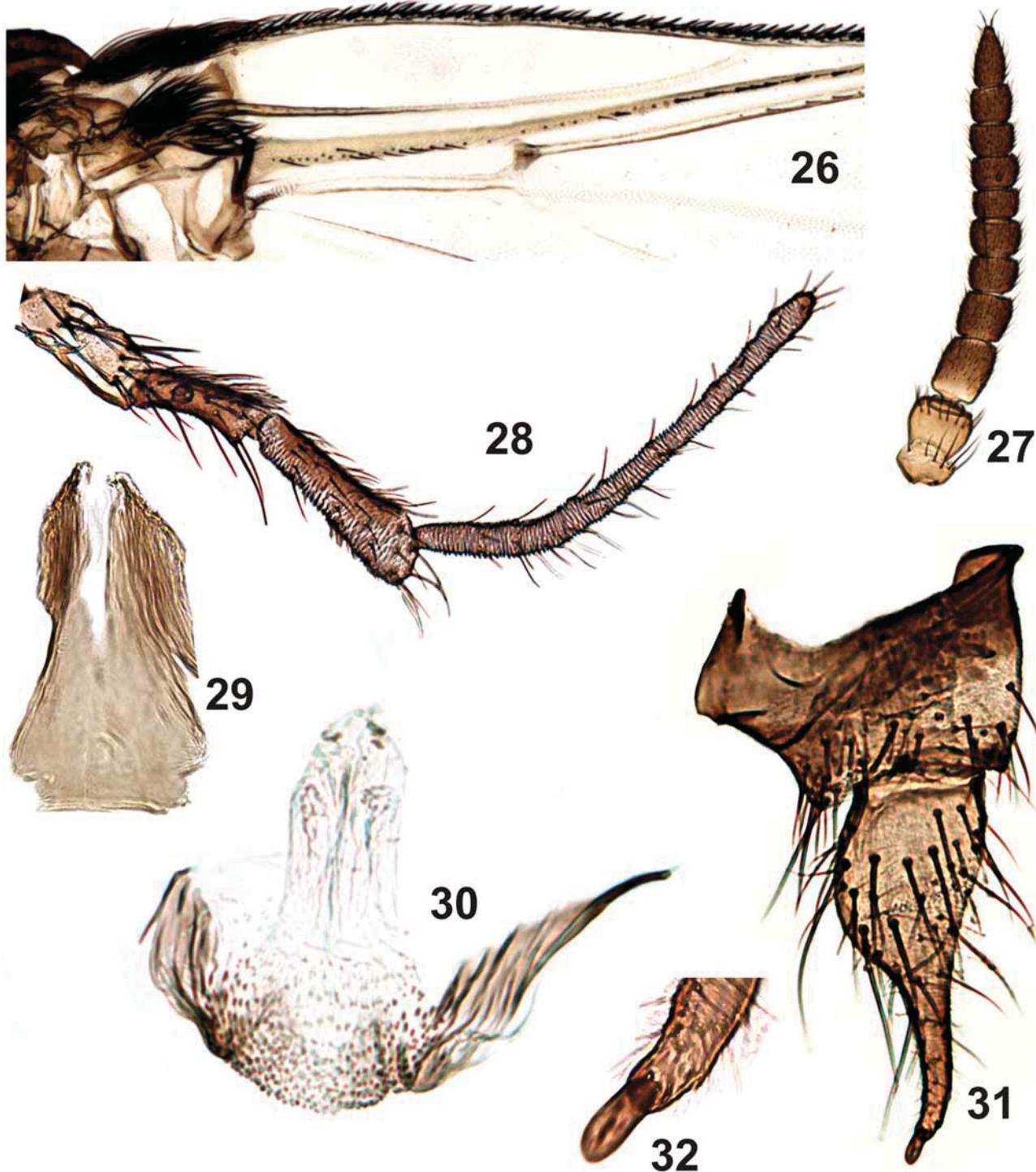


24



25

**FIGURES 17–25.** Male. 17–19. *Simulium guianense* (Diptera: Simuliidae). 17. Scutum, dorsal view, anterior illumination. 18. Scutum, dorsal view, posterior illumination. 19. Scutum, dorso-lateral view. 20–25. *Simulium litobranchium* n. sp. 20–22. Goias state population. 20. Scutum, dorsal view, anterior illumination. 21. Scutum, dorsal view, posterior illumination. 22. Scutum, dorso-lateral view. 23–25. Minas Gerais state population. 23. Scutum, dorsal view, anterior illumination. 24. Scutum, dorsal view, posterior illumination. 25. Scutum, dorso-lateral view.

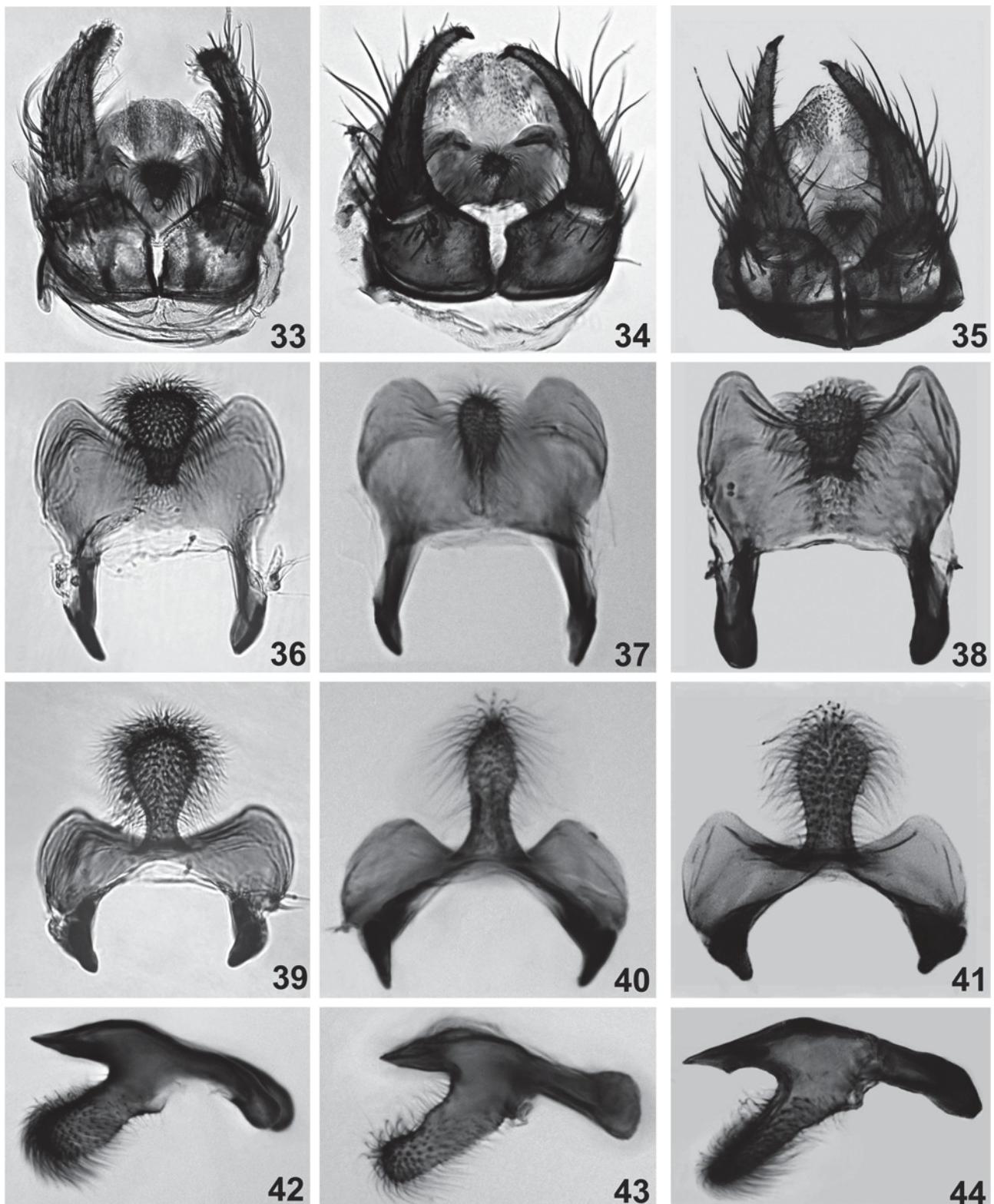


**FIGURES 26–32.** Male of *Simulium litobranchium* n. sp. (Diptera: Simuliidae). 26. Wing base. 27. Antenna. 28. Maxillary palp. 29. Median sclerite. 30. Aedeagal membrane and paramere. 31. Gonocoxite and gonostylus, mounted between slide and coverslip, lateral view. 32. Spinule at the apex of gonostylus.

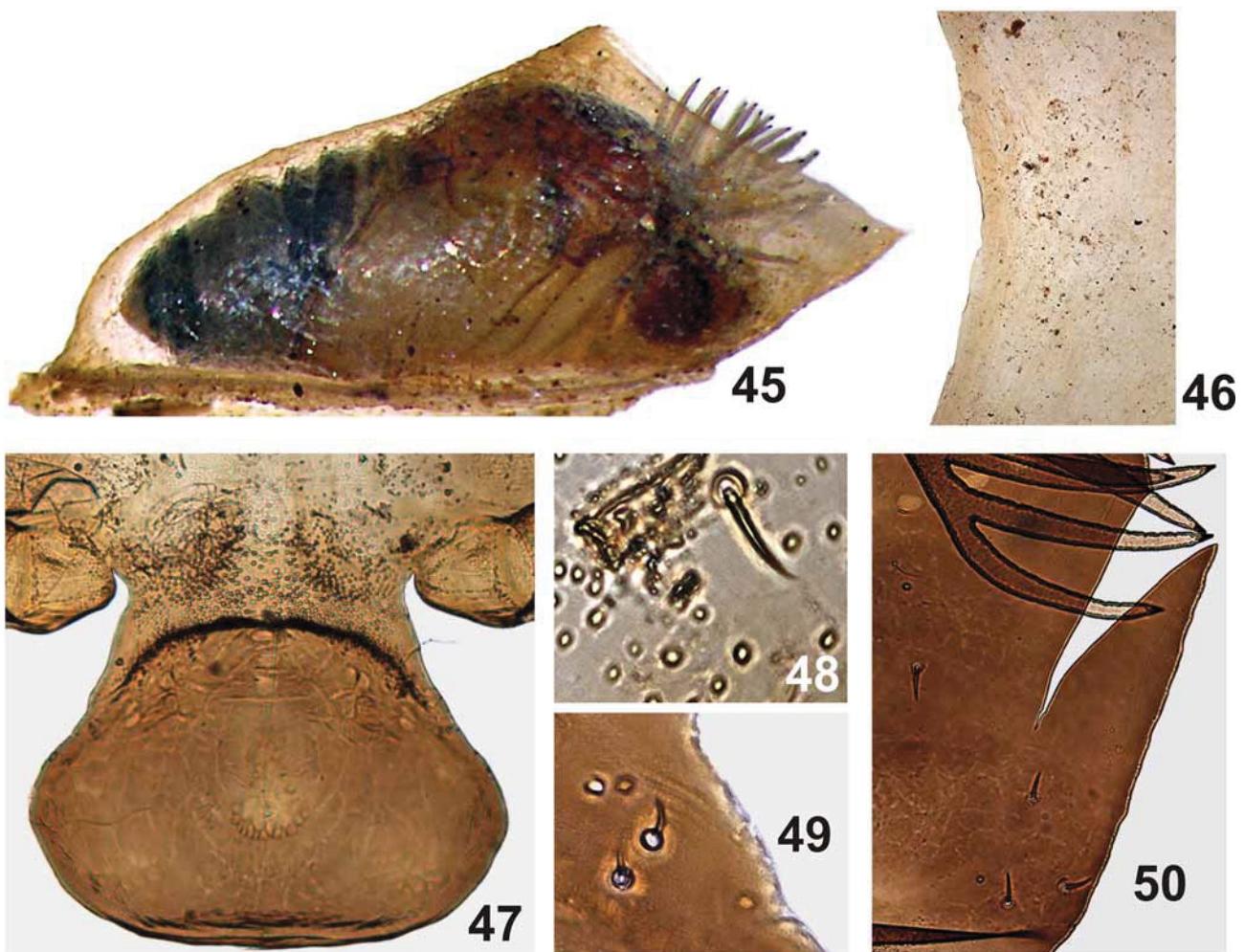
MALE (Figs 20–32, 35, 38, 41, 44). General body color black, body mean length, 1.7 mm ( $SD = 0.14$ ,  $n = 5$ ); lateral thorax mean length (from neck to anterior region of wing insertion) 0.5 mm ( $SD = 0.06$ ,  $n = 5$ ). Wing mean length 2.0 mm ( $SD = 0.09$ ,  $n = 10$ ), mean width 1.0 mm ( $SD = 0.025$ ,  $n = 10$ ).

Antenna (Fig. 27) mean length 0.51 mm ( $SD = 0.04$  mm,  $n = 5$ ); scape and pedicel brownish yellow, subsequent flagellomeres increasingly dark brown. Palpus (Fig. 28) brown, palpomere V about 2.4–3.5 times as long as palpomere III and 2.2–2.8 times as long as palpomere IV; sensory vesicle small, subspherical, 1/4–

1/5 the length of palpomere III length. Scutum black (Figs 20–25), covered with golden hairs and a pair of dorso-lateral silver spots (Figs 20–25). Anepisternum and katepisternum brown with silver pruinosity.



**FIGURES 33–44.** Male terminalia and ventral plate. *Simulium duodenicum* (Diptera: Simuliidae). 33. Terminalia, ventral view. 36. Ventral plate, ventral view. 39. Ventral plate, in ventral view tilted dorsally. 42. Ventral plate, lateral view. *Simulium guianense*. 34. Terminalia, ventral view. 37. Ventral plate, ventral view. 40. Ventral plate, in ventral view tilted dorsally. 43. Ventral plate, lateral view. *Simulium litobranchium n. sp.* 35. Terminalia, ventral view. 38. Ventral plate, ventral view. 41. Ventral plate, in ventral view tilted dorsally. 44. Ventral plate, lateral view. [Modified from Pepinelli *et al.* (2005)]

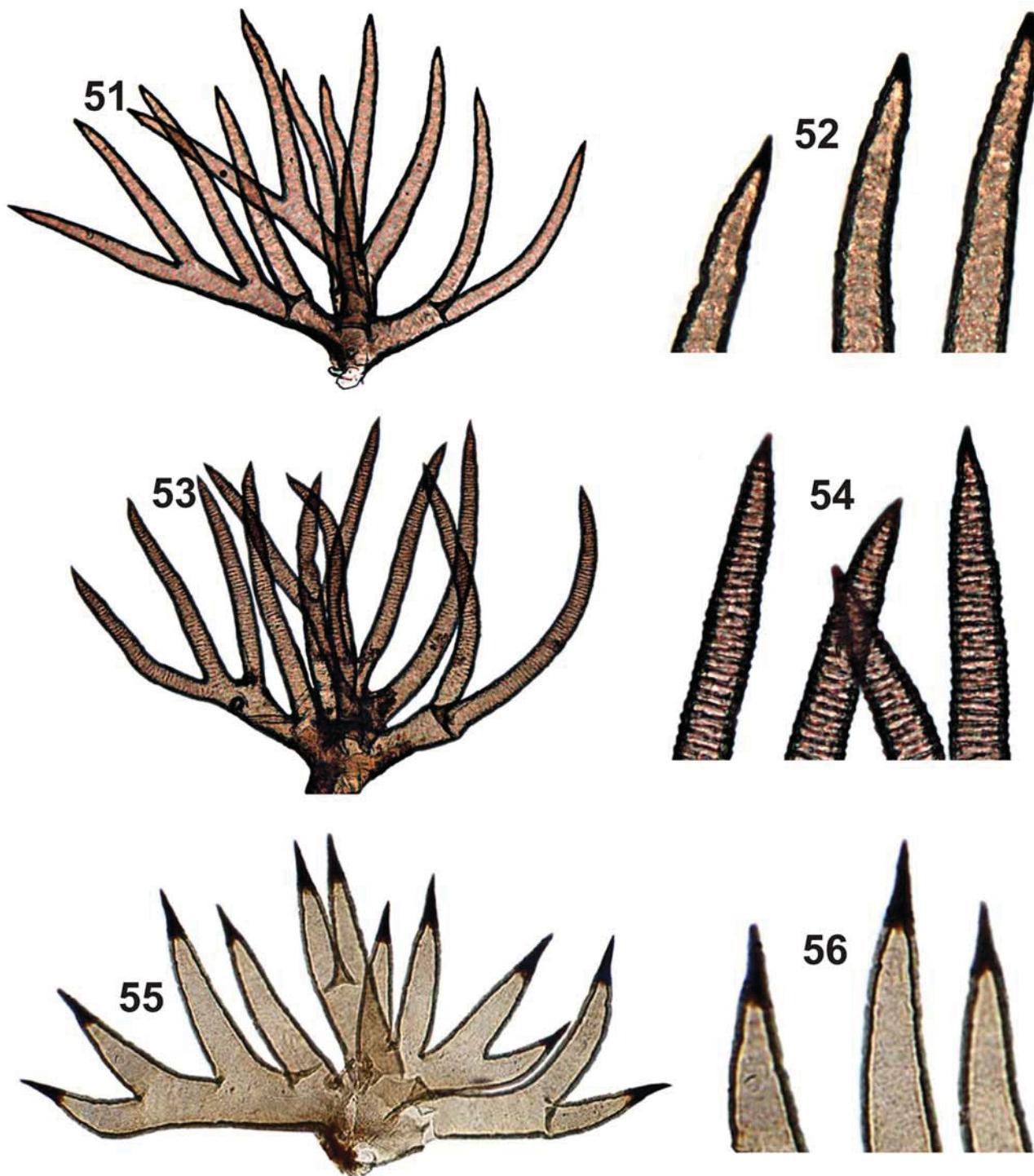


**FIGURES 45–50.** Pupa of *Simulium litobranchium* n. sp. (Diptera: Simuliidae). 45. Pupa inside cocoon, lateral view. 46. Middle region of the anterior margin of the cocoon, in dorsal view. 47. Male cephalic plate. 48. Head capsule frontal trichome. 49. Head capsule dorsal trichome. 50. Thoracic trichomes.

Scutellum brown with thin golden hairs. Postnotum brown with silver pruinosity. Wing similar to the female, except in Sc, which is mostly bare (Fig. 26); only one of 12 males examined had hair-like setae on Sc. Legs with same color pattern as female, except that, in general, the male legs are darker. Halter yellow. Abdominal basal fringe with thin, long brown hairs; tergites II, V–VII with lateral silver pruinosity. Gonocoxite and gonostylus (Figs 31, 35) brown, gonocoxite wider than long; gonostylus more than twice the gonocoxite length, longer than wide, bearing one spinule at the apex (Figs 31, 32). Ventral plate (Figs 38, 41, 44), in ventral view, subrectangular, with a median anterior projection enlarged distally and covered with setae. Median sclerite (Fig. 29) subrectangular, with mid-apical region lightly sclerotized. Paramere weakly sclerotized, poorly developed and aedeagal membrane with spicules as in Figure 30.

PUPA (Figs 45–52, 57, 58). Mean body length 3.0 mm ( $SD = 0.2$ ,  $n = 5$ ). Cocoon (Fig. 45) boot-shaped, thick and hard, without central projections (Fig. 46). Mean length along cocoon dorsal surface 2.0 mm ( $SD = 0.2$ ,  $n = 5$ ). Head, cephalic plate with 1 + 1 short, thick, simple frontal trichome (Fig. 48) and 2 + 2 short, thick, simple dorsal trichomes (Fig. 49). Round tubercles present on the ventral region of cephalic plate, absent on the dorsal region. Thorax without tubercles on the dorsal anterior region; dorsal posterior region, with majority of tubercles pointed, but some rounded in the area below the tracheal trunk of gill filaments. Thorax with 5 + 5 short, thick simple trichomes (Fig. 50), rarely 1 + 1 bifid; 1 + 1, thick, longer lateral trichome. Gills with 12 short, thick, rigid filaments, distributed in a three-dimensional pattern with tips pointed and sclerotized, without spicules in annular arrangement (Figs 51, 52). Abdomen as in Figures 57 and 58. Tergite I with 1 + 1 sublateral setae. Tergite II with 4 + 4 stout setae (Fig. 57a), 2 + 2 thin sublateral setae and many small, tubercles

distributed, especially, in the anteromedian region of the tergite. Tergites III and IV each with 4 + 4 anteriorly directed hooks on posterior margin (Figs 57b, 57c). Tergites VI–IX with comb-like groups of fine posteriorly directed spines on anterior margin (Fig. 57d). Sternites III–VIII with anterior medial comb-like groups of microspines (Fig. 58a). Sternites V–VII with 2 + 2 stout, bifid hooks (Figs 58b, 58c, 58d).



**FIGURES 51–56.** Pupal gill filaments and apex of gills. Figs 51–52. *Simulium litobranchium* n. sp. (Diptera: Simuliidae). Figs 53–54. *Simulium guianense*. Figs 55–56. *Simulium duodenicum*.

LARVA (last instar) (Figs 59–76). Body mean length 4.3 mm ( $SD = 0.2$ ,  $n = 5$ ); head capsule, lateral mean length 0.4 mm ( $SD = 0.02$ ,  $n = 5$ ).

General coloration varying from light to dark grayish green (Figs 59–62) (in Carnoy's solution). Head cuticle with small simple setae. Frontoclypeal apotome, in light colored larvae, with positive pattern as in Figure 63 and, in dark colored larvae, with negative pattern as in Figure 65. Cervical sclerites small, elliptical, free in the membrane, each with a thin, elongated sclerite anteriorly (Figs 67, 69). Postgenal cleft widest at medial region and with anterior end subtriangular (Figs 68, 70), area inside cleft irregularly pigmented (Figs 64, 66). Postgenal bridge 0.3–0.6 times as long as hypostoma (Figs 68, 70). Antenna as long as labral fan stalk; distal and proximal articles smaller than medial (proportions of articles, proximal to distal, excluding cone sensillum, 1:1.4–1.75: 0.8–0.9) (Fig. 72). Labral fan with 42–56 primary rays (Mean = 49, SD = 5, n = 7). Hypostoma with pigmented anterior margin (Fig. 71) with median tooth and sublateral teeth not well differentiated, 2 + 2 sublateral teeth with 1 – 2 small serrations; 1 + 1 strong lateral tooth and 1 – 2 paralateral teeth, with 5 – 7 sublateral setae per side. Mandibular teeth: one apical, two small external; three subapical; 6 – 7 internal teeth; one mandibular serration and one small mandibular sensillum (*sensu* Craig & Craig, 1986). Gill histoblast dissected with 12 smooth filaments, with sclerotized and pointed apex. Body covered with ovoid setae (Fig. 73); ventral tubercles absent (Figs 59–62). Abdomen with 1 + 1 dorso-lateral tubercles (Fig. 76), on segments 1 – 5 (Figs 59–61), or 1 – 6 (Fig. 62), varying from well to poorly developed (Figs 59–62), especially in the population from Minas Gerais. Anterodorsal arms of anal sclerite 0.5 times the length of posteroventral arms (Fig. 74), presence of thin and few enlarged setae near the arms. Posterior circlet bearing 139–166 rows (Mean = 153, SD = 10, n = 9) with 22–26 hooks (Mean = 24, SD = 2, n = 9). Rectal papillae with three branches, each with approximately 18 – 31 digitiform lobes (Fig. 75); mean number ( $\pm$ SD) on each branch = 24( $\pm$ 3) + 24( $\pm$ 2) + 28( $\pm$ 4), n = 9.

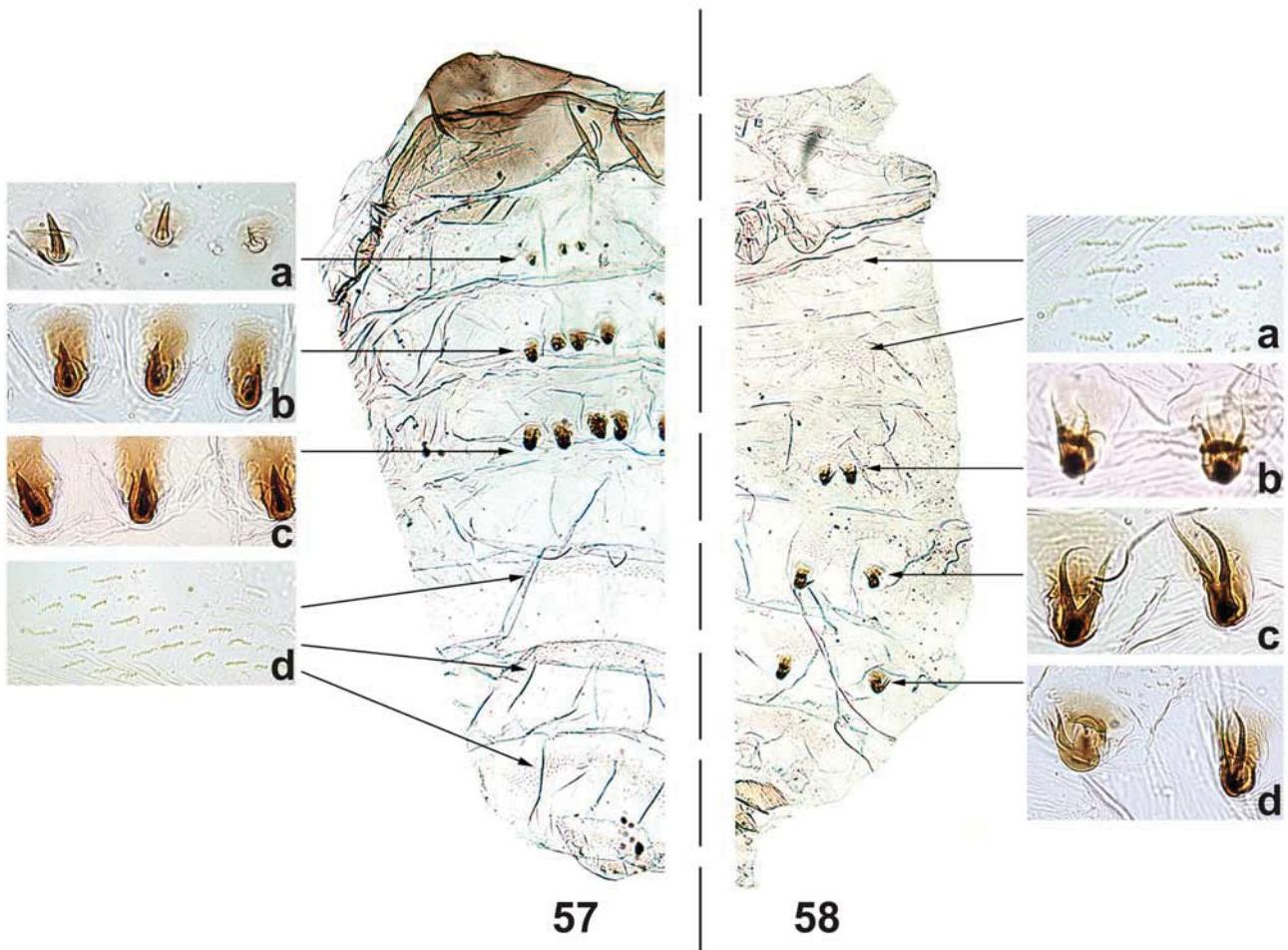
**TYPES.** **Holotype** – male (M), pinned, collected in the Ponte de Pedra River, Montividiu municipality, Goiás, 17°10'S, 50°50'W, 671 masl, collectors N. Hamada, M. Pepinelli & V. Landeiro, 21/05/2006 (INPA).

**Paratypes** - same locality, date and collectors as holotype, 8 pupae (P) and 9 larvae (L) in 80% ethanol; 5 P and 8 L mounted on slide; 3 females (F) and 5 M pinned with their pupal exuviae in glycerine; 5 M and 2F mounted on slide together with its pupal exuviae (INPA). Same locality as holotype, collectors N. Hamada & M. Pepinelli, 15/07/2004, 2 F and 3 M (INPA). Same locality, date and collectors as holotype, 8 P and 9 L in 80% ethanol, 3 F and 4 M pinned with their pupal exuviae in glycerine (MZUSP). Montividiu River, Montividiu municipality, Goiás, 17°26'S, 51°10'W, 779 masl., 21/05/2006, collectors N. Hamada, M. Pepinelli & V. Landeiro, 21/05/2006, 2 F, pinned with its pupal exuviae (INPA). Santo Antônio River, Delfinópolis municipality, Minas Gerais, 20°15'S, 46°51'W, 726 masl, collectors N. Hamada & M. Pepinelli, 18/09/2005 (INPA), 15 P and 10 L in 80% ethanol; 1 P and 5 L mounted on slide, 4 F and 5 M, pinned with their pupal exuviae (INPA); 15 P and 10 L in 80% ethanol, 4 F and 5 M, pinned with their pupal exuviae (MZUSP).

**Etymology:** The species name derived from *lito* (G) = smooth and *branchium* (G) = gill, in reference to smooth appearance of the gill filaments due to the lack of spicules in annular arrangements.

**Taxonomic discussion.** The new species described in this paper can be placed in the subgenus *Thrysopelma* or *Trichodagmia*, depending on which black fly classification scheme is being followed. This situation occurs because the validity of the subgenus *Thrysopelma* Enderlein is debated (e.g. Miranda-Esquível & Coscarón 2001, Shelley *et al.* 1997). *Thrysopelma* is considered synonymous with *Trichodagmia* Enderlein in the inventory of world black flies by Adler & Crosskey (2009). In this study we are following the Miranda-Esquível & Coscarón (2001) classification scheme.

Female scutal pattern of *S. litobranchium* n. sp. is similar to most of the other described species in *S. (Thrysopelma)*; the exceptions are *S. scutistriatum* and *S. perplexum*, but the new species can be distinguished from these by the shape of the anal lobe (Figs 12, 13). The only species from which it cannot be distinguished based on this character is *S. duodenicornutum* Hamada, Pepinelli & Trivinho-Strixino, which also has the internal side of the anal lobe concave in the medial region, forming two short projections of similar width. However, the female of the new species can be distinguished from that of *S. duodenicornutum*, among other characters, by the presence of hair-like setae on the Sc, by the shape of the tarsal claws (which are shorter and more curved than those of *S. duodenicornutum*) and by having fronto-ocular triangle longer and more pointed than that of *S. duodenicornutum*. The female of *S. jeteri* (Py-Daniel, Darwich, Mardini, Strieder & Coscarón) was incompletely described (Py-Daniel *et al.* 2005); therefore, morphological comparisons are not possible.



**FIGURES 57–58.** Pupa of *Simulium litobranchium* n. sp. (Diptera: Simuliidae). 57. Abdomen, dorsal view. 57a–c: setae and hooks on tergites II–IV. 57d: group of spine combs on tergites VI–IX. 58. Abdomen, ventral view. 58a: group of spine combs on sternites III–VIII. 58b–d: hooks on sternites V–VII.

Male of *S. litobranchium* n. sp. is similar to most of the males in the *S. (Thrysopelma)* subgenus, with the exception of *S. duodenicornium*, *S. scutistriatum* and *S. perplexum*, which do not have silver spots on the scutum. The male of the new species and those of *S. itaunense*, *S. orbitale* and *S. duodenicornium* can be differentiated from the male of *Simulium guianense* based on the fact that the latter has a more elaborated silver pattern on the scutum (Figs 17–19), while in the other species, the pattern is basically a pair of lateral silver spots (Figs 20–25). Males of *S. jeteri* were incompletely described (Py-Daniel *et al.* 2005); therefore, morphological comparisons are not possible.

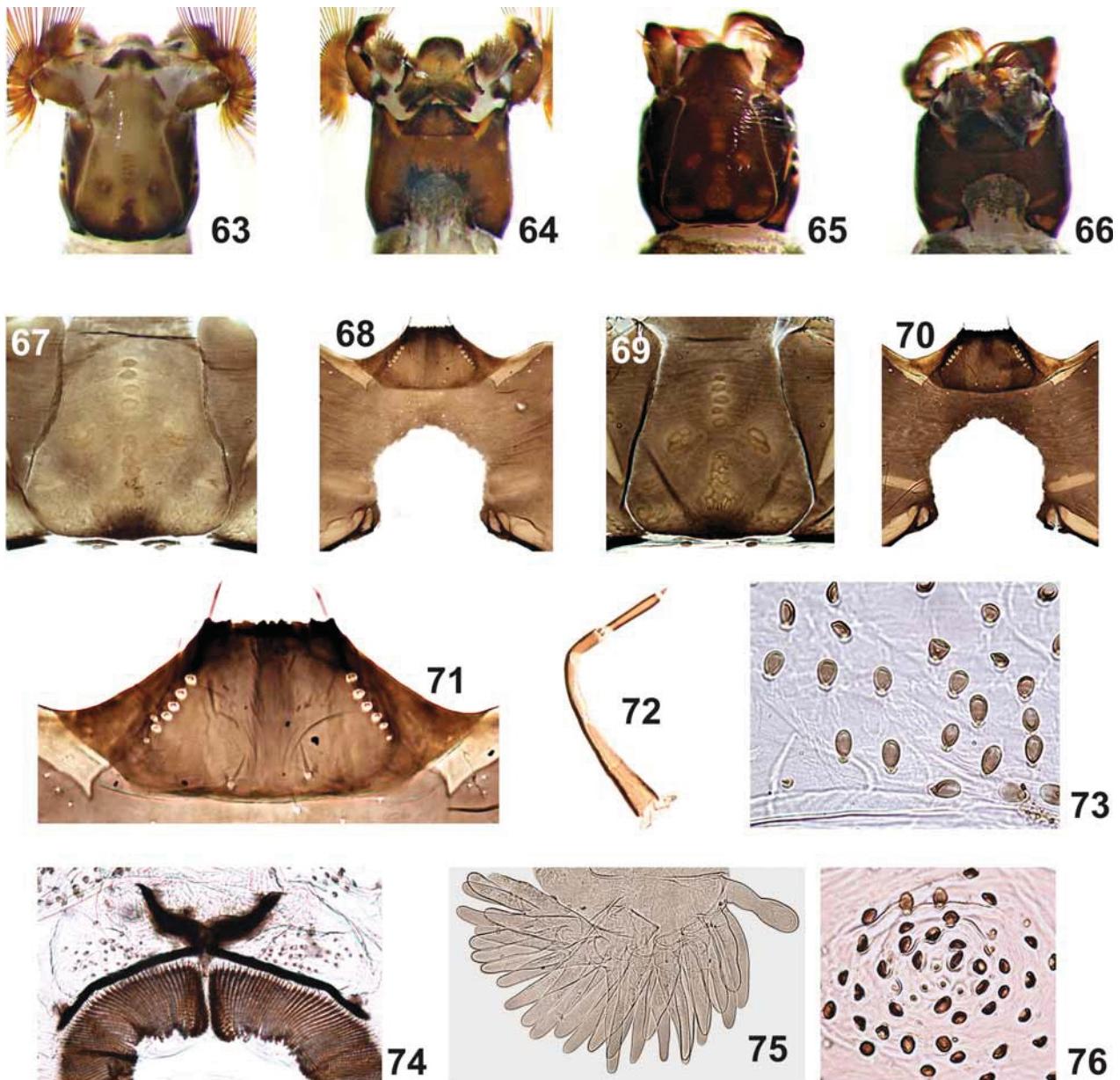
The male genitalia of *S. guianense*, *S. duodenicornium* and *S. litobranchium* n. sp. are very similar. However, morphological difference can be visualized in the ventral plate (Figs 33–44). Pepinelli *et al.* (2005) mistakenly stated that the ventral plates of *S. duodenicornium* and *S. guianense*, in Figures 27 and 28 were shown in dorsal view; instead, the ventral plates in the above-cited figures were shown in ventral view tilted dorsally. According to Crosskey (1990), due to the diversity of shapes of this structure in Simuliidae in general and the similarity of the characteristics of this plate between related species, the ventral plate is one of the best characters for distinguishing species and groups of species. The *S. litobranchium* n. sp. ventral plate can be distinguished from that of *S. guianense* by the width of the apical region of the projection, which is larger in the former than in the latter species (Figs 37, 38). But this structure is very similar in the new species and in *S. duodenicornium* (Figs 39, 41); the only difference can be visualized in the anterolateral region of the ventral plate, which is more elongated in the new species than in *S. duodenicornium* (Figs 39, 41).

At the pupal stage, the number, shape and disposition of the gill filaments of *S. litobranchium n. sp.* are similar to those of five species in the *S. (Thrysopelma)*: *S. guianense*, *S. perplexum*, *S. duodenicornium*, *S. scutistriatum* and *S. hirtipupa*. However, except for *S. duodenicornium* and *S. scutistriatum*, all of these species have spicules or projections in an annular arrangement on the gill filament surface (Shelley *et al.* 1989, Shelley *et al.* 1997, Pepinelli *et al.* 2005). Pupae of the new species can be distinguished from those of *S. hirtipupa* because the latter has a cocoon with irregular protuberances and a large number of spines on the cephalic and thoracic region. The pupa of new species can be distinguished from that of *S. scutistriatum* by the cocoon shape of the latter species, which has the anteroventral margin more elongated; additionally, the latter species has a cephalic plate with several acuminate tubercles. The pupa of the new species can be distinguished from that of *S. duodenicornium* by the presence, in the latter species, of small tubercles distributed evenly on tergite II and by the width of the gill filaments, which are thicker basally in the latter species (Pepinelli *et al.* 2005).

The larvae of *S. litobranchium n. sp.* are similar to those of most species in the subgenus *S. (Thrysopelma)*, with ovoid setae covering the body cuticle (exceptions are *S. scutistriatum* and *S. perplexum*). The new species has paired dorsal tubercles, varying from weakly to strongly developed on the 1<sup>st</sup> to the 5<sup>th</sup> or 6<sup>th</sup> abdominal segment (some larvae do not have the paired tubercles on the 6<sup>th</sup> segment). The other characteristic that can be used to easily distinguish the new species from the other in *S. (Thrysopelma)* is the gill histoblast of last-instar larvae: after dissection, the same gill pupal characteristic can be observed.



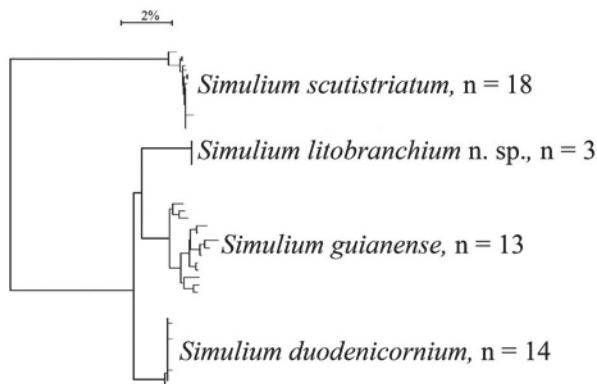
**FIGURES 59–62.** Lateral view of *Simulium litobranchium n. sp.* larvae (Diptera: Simuliidae) showing color variation and variation in the development and number of paired dorsal tubercles.



**FIGURES 63–76.** Larva of *Simulium litobranchium* n. sp. (Diptera: Simuliidae). 63–70. Variation in the head color. 63. Head capsule, dorsal view. 64. Head capsule, ventral view. 65. Head capsule, dorsal view. 66. Head capsule, ventral view. 67. Dorsal view of cephalic apotome and cervical sclerites. 68. Postgenal cleft, ventral view. 69. Dorsal view of cephalic apotome and cervical sclerites. 70. Postgenal cleft, ventral view. 71. Hypostoma. 72. Antenna. 73. Lanceolated setae on body cuticle. 74. Anal sclerite. 75. Papillae of one of the three lobes of rectal papilla. 76. Dorsal tubercles on cuticle of abdominal segment.

The synonyms of *Simulium pintoi* d'Andretta & d'Andretta and *S. ortizi* Ramírez-Pérez with *S. guianense* and of *S. brasiliense* (Enderlein) with *S. orbitale* are well established and widely accepted (Adler & Crosskey, 2009). *Simulium pintoi* and *S. ortizi* can be distinguished from the new species using the same characters used to distinguish it from *S. guianense*, especially female genitalia and male scutal pattern.

*Simulium brasiliense* was succinctly described based on the female and was established as a synonym of *S. orbitale*; since the anal lobe of *S. litobranchium* n. sp. has its internal side concave in the medial region, forming two short projections of similar width it cannot be misidentified with the species cited above because the females of these species have a tail-like projection on the membranous part of the anal lobe. Also, the hypogynial valves are rounded in the new species while in the other species cited above they are subovoidal, longer than wide.



**FIGURE 77.** A Kimura 2-parameter neighbor-joining tree for 4 nominal species of Neotropical black flies *S.* (*Thrysopelma*) subgenus. Scale bar = 2%

**DNA barcoding.** Simuliidae is a taxonomically challenging family because these insects are small in size and structurally homogeneous, and because of the presence of reproductively isolated (but morphologically indistinguishable) sibling species (Adler *et al.* 2004). For this reason, knowledge about all life stages is important, and the use of a multifaceted approach incorporating both morphological and DNA-sequence data, such as DNA barcoding, is critical for species recognition.

In the present study, DNA barcoding discriminated species within the subgenus *S.* (*Thrysopelma*) (Fig. 77). Mean intraspecific genetic divergence for the four species analyzed was 0.51%. *Simulium guianense* had a maximum intraspecific divergence of more than 2.5% (Table 1). According to Rivera & Currie (2009), in the Nearctic region, the mean intraspecific divergence for 58 black fly species was 0.76%, and the maximum intraspecific divergence value was 3.84% [observed in *Simulium rostratum* (Lundström)]. The high divergence (> 4%) in the sequences of the COI gene (Table 2) among the four species we analyzed suggests that they are different species; DNA barcoding, therefore, corroborates the morphological data we provided to establish that *Simulium litobranchium* n. sp. is not a variant of *S. guianense*, as indicated by Shelley *et al.* (2002).

**TABLE 2.** Distance matrix between four species of the subgenus *S.* (*Thrysopelma*), based on Kimura 2-parameter pairwise distances.

Species	1	2	3	4
<i>S. litobranchium</i> (1)				
<i>S. duodenicornium</i> (2)	0.04			
<i>S. guianense</i> (3)	0.04	0.04		
<i>S. scutistriatum</i> (4)	0.14	0.13	0.14	

**Bionomics.** The Paraná River, which is the main water course in this hydrographic basin, has as major tributaries, among others, the Rio Grande, Paranaíba, Tietê, Paranapanema and Iguaçu rivers. Despite the heavy anthropogenic impact caused by agriculture in the case of the two rivers in Goiás state, *Simulium litobranchium* n. sp. was collected in abundance. *Simulium litobranchium* n. sp. larvae and pupae were collected in three rivers, varying in width from 30 to 40 meters, two in Goiás (Paranaíba River basin) and one in Minas Gerais state (Rio Grande basin). *Simulium guianense* was also collected in these same hydrographic basins, but in larger tributaries (more than 150 m in width). The streams where the new species was collected had a streambed composed of sand, small stones and boulders. The water temperature ranged from 17 to 19° C, pH from 6.5 to 7.3, and electrical conductivities were low (below 15µ/cm). Larvae and pupae were collected on leaves of plants in the family Podostemaceae, submerged tree branches and leaves of riparian vegetation and leaves of submerged grass. The female of the new species was not collected biting humans during the fieldwork.

## Acknowledgments

This study received partial financial support from CNPq/MCT and INPA/MCT. We would like to thank Dr. Paul Hebert and the Canadian Centre for DNA Barcoding for supporting the barcoding studies. Victor Lemes Landeiro helped in the fieldwork. Nelson Hamada provided the vehicle that allowed us to sample the study area. The first, second and third authors received fellowships from CNPq, Fapesp and Fapeam, respectively. Marcio Cutrim made the adult scutal plates presented in this paper. Philip M. Fearnside reviewed the manuscript.

## References cited

- Adler, P.H., Currie, D.C. & Wood, D.M. (2004) *The Black Flies (Simuliidae) of North America*. Cornell University Press, Ithaca, New York, 941 pp.
- Adler, P.H. & Crosskey, R.W. (2009) World blackflies (Diptera: Simuliidae): a comprehensive revision of the taxonomic and geographical inventory. <http://entweb.clemson.edu/biomia/pdfs/blackflyinventory.pdf>.
- Avise, J.C. (1994) *Molecular markers, Natural History and Evolution*. Chapman & Hall. New York, London. 511 pp.
- Caterino, M.S., Cho, S. & Sperling, F.A.H. (2000) The current state of insect molecular systematics: a thriving tower of Babel. *Annual Review of Entomology*, 45, 1–54.
- Combine, Z.M. (2007) Public Domain Software by Alan Hadley. Release date 1<sup>st</sup> of December 2007.
- Craig, D.A. & Craig, R.E.G. (1986) Simuliidae (Diptera: Culicomorpha) of Rarotonga, Cook Islands, South Pacific. *New Zealand Journal of Zoology*, 13, 357–366.
- Crosskey, R.W. (1990) *The natural history of blackflies*. John Wiley and Sons, Chichester, England. 711 pp.
- Hebert, P.D.N., Cywinski, A., Ball, S.L. & deWaard, J.R. (2003) Biological identifications through DNA barcodes *Proceedings of the Royal Society of London. Series B, Biological Sciences*, 270, 313–321.
- Kimura, M. (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide-sequences. *Journal of Molecular Evolution*, 16(2), 111–120.
- Luna Dias, J.P.A., Hernández, L.M., Maia-Herzog, M. & Shelley, A.J. (2004) *Simulium lobatoi*, a new species of blackfly (Diptera: Simuliidae) from the states of Mato Grosso and Goiás, Brasil. *Memórias do Instituto Oswaldo Cruz*, 99, 37–43.
- Miranda-Esquivel, D.R. & Coscarón, S. (2001) Cladistic analysis of *Simulium (Trichodagmia)* and *Simulium (Thrysopelma)* (Diptera: Simuliidae). *Zoological Journal of Linnean Society*, 132, 429–439.
- Pepinelli, M., Hamada, N. & Trivinho-Strixino, S. (2005) *Simulium (Thrysopelma) duodenicornium* n. sp., a new black fly species (Diptera: Simuliidae) from the Southeast region of Brazil. *Zootaxa*, 1040, 17–29.
- Py-Daniel, V., Magni-Darwich, S., Mardini, L.B., Strieder, M.N. & Coscarón, S. (2005) Simuliidae (Diptera, Culicomorpha) no Brasil – XII sobre o gênero *Thrysopelma* Enderlein, 1934: descrição de *T. jeteri* sp.n., considerações sobre o cibário e chave para as pupas. *Acta Amazonica*, 35(4), 463–472.
- Rivera, J. & Currie, D.C. (2009) Identification of Nearctic black flies using DNA barcodes (Diptera: Simuliidae). *Molecular Ecology Resources*, 9 (Suppl. 1), 224–236.
- Roderick, G.K. (1996) Geographic structure of insect populations: Gene Flow, Phylogeography, and their uses. *Annual Review of Entomology*, 41, 325–352.
- Sabrosky, C.W. (1966) Mounting insects from alcohol. *Bulletin of the Entomological Society of America*, 12, 349.
- Shelley, A.J., Maia-Herzog, M., Luna Dias, A.P.A. & Couch, C.A. (1989) Description of the adults and pupa of *Simulium (Trichodagmia) perplexum*, new species (Diptera: Simuliidae). *Memórias do Instituto Oswaldo Cruz*, 84, 343–349.
- Shelley, A.J., Lowry, C.A., Maia-Herzog, M., Luna Dias, A.P.A. & Moraes, M.A.P. (1997) Biosystematic studies on the Simuliidae (Diptera) of the Amazonia onchocerciasis focus. *Bulletin of Natural History Museum of London (Entomology)*, 66, 1–121.
- Shelley, A.J., Hernández, L.M., Maia-Herzog, M. & Luna Dias, A.P.A. (2002) Morphological variation in the larva of *Simulium guianense* s.l. Wise (Diptera, Simuliidae), a primary vector of human onchocerciasis in Brazil. *Entomologist's Monthly Magazine*, 138, 95–102.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24, 1596–1599.